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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Apoptosis, or programmed cell death, is an energy-dependent process of cell suicide shown to be critical in many physiological and pathologic venues including tumor development. The gene *reaper* from *Drosophila melanogaster*, has proven roles in radiation-induced and developmental apoptosis, and is p53-induced. Using a cell-free extract system, our lab has identified a 150 kDa protein from *Xenopus laevis* called *scythe* that is required for reaper-induced apoptosis. The interaction of reaper with scythe liberates a soluble factor (SCF) that induces apoptosis by effecting the release of cytochrome c from mitochondria, a critical step in activating apoptosis in many systems. Our purpose is to identify and characterize SCF, and to evaluate its therapeutic potential.

We have preliminary evidence that SCF at least in part contains the molecular chaperone Hsp70. Upon interaction with reaper, scythe releases Hsp70 which then acts at the mitochondrial surface to effect the redistribution of cytochrome c. As expected, blocking the chaperone activity of Hsp70 prevents reaper-induced apoptosis. We have successfully reconstituted this process *in vitro* using purified components. The next step in our research will be to identify the mitochondrial substrate(s) of Hsp70 necessary for reaper-induced apoptosis.

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INTRODUCTION

Apoptosis, or programmed cell death, is an energy-dependent process of cell suicide shown to be critical in many physiological and pathologic venues including tumor development. The gene *reaper* from *Drosophila melanogaster*, has proven roles in radiation-induced and developmental apoptosis, and is p53-induced. Using a cell-free extract system, our lab has identified a 150 kDa protein from *Xenopus laevis* called *scythe* that is required for reaper-induced apoptosis. The interaction of reaper with scythe liberates a soluble factor (SCF) that induces apoptosis by effecting the release of cytochrome c from mitochondria, a critical step in activating apoptosis in many systems. Therefore, Scythe sequesters a novel cytochrome c releasing factor (SCF) that is essential for reaper induced apoptosis. We hypothesize that SCF (or its human homologue), like Reaper itself, will be sufficient to induce apoptosis in human breast cancer cells, and may provide a particular therapeutic benefit in situations where the p53 responsive pathway is otherwise compromised. We have proposed to identify, clone, and characterize SCF, and then to test the therapeutic potential of SCF by assaying its ability to cause apoptosis in a variety of model systems, including cultured human cells.

BODY

The period of support (1 August 2001 - 31 July 2002) corresponds to months 1-12 of the grant per the approved Statement of Work. This time period is entirely contained within Task one:

Task 1 To identify the *Scythe*-interacting protein (SCF) responsible for inducing cytochrome c release from mitochondria (months 1-14):

a. utilize recombinant wild-type *Scythe* to affinity purify SCF from *Xenopus* egg extract. (months 1-2)
b. test effectiveness of reaper peptide in releasing SCF from scythe immunoprecipitate (months 1-2)
c. test importance of *Scythe* BH_3 domain on SCF release and activity (months 1-4)
d. fractionate released proteins to purify SCF (months 2-12)
e. perform caspase and mitochondrial cytochrome c release assays to track SCF throughout purification (months 2-12)
f. microsequence and identify SCF (months 12-14)

We took a multi-pronged approach to identify SCF. The first approach involved the biochemical purification of SCF from crude Xenopus extracts (tasks 1a-1b, 1d-1f above). To this end, we (previously) had developed an *in vitro* assay to track SCF activity. In this assay, we took fractions containing SCF activity and mixed them with mitochondria purified from crude *Xenopus* eggs. As shown in Fig. 1 below, when 6xHis-tagged scythe is incubated with crude egg extract and then precipitated on Ni-NTA resin,

Reaper addition will generate SCF activity (assayed as release of cytochrome c, detected by Western blot with anti-cytochrome c antibody).

Next, a variety of resins were employed to trap SCF from the soluble fractions generated in Fig. 1. Several of the resins (Mono Q, DEAE) successfully depleted SCF activity (Fig. 2).

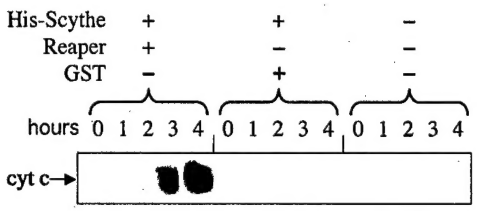

Fig. 1: His-Scythe traps SCF

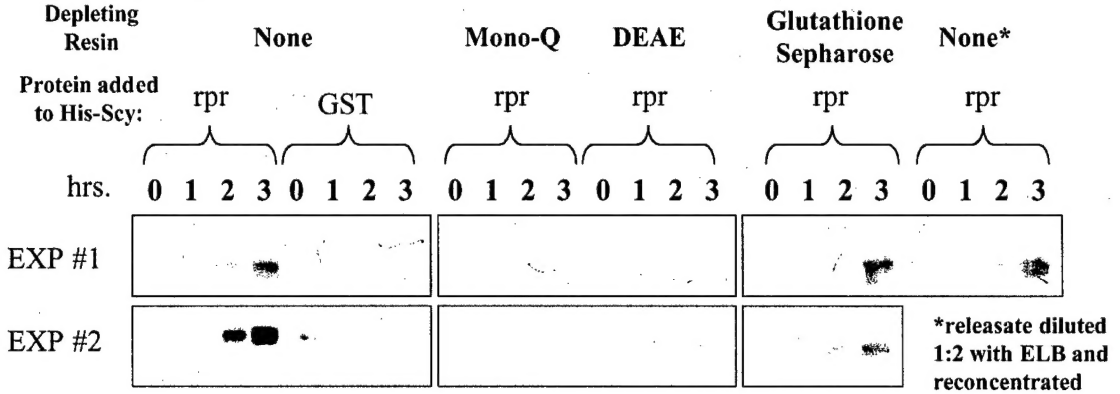

Fig. 2: Trapping of SCF on resins

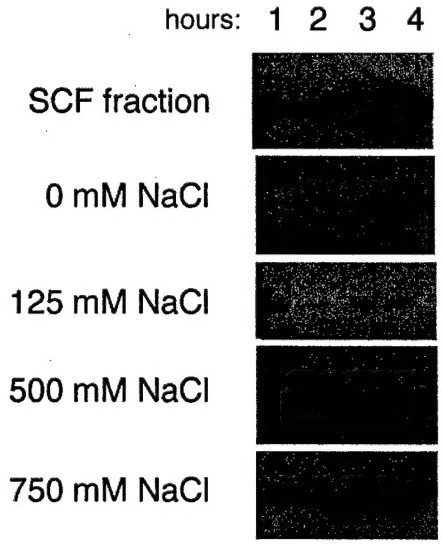

Fig. 3: NaCl bump from DEAE resin

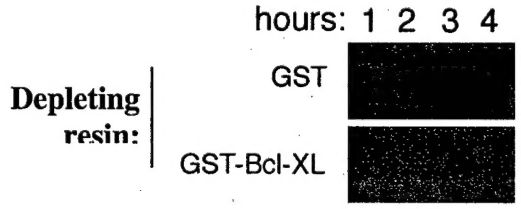

Fig. 4 Bcl-XL depletion of SCF fraction

Serial salt elutions from DEAE were then performed. SCF activity was eluted from DEAE using 0.5M and higher NaCl (Fig 3). At the same time, affinity resins were produced to attempt depletion of specific proteins from the SCF-containing fractions. As shown in Fig. 4, Bcl-XL, a well-characterized member of the Bcl-2 family, successfully removes SCF from the soluble fraction. This is interesting in light of the published observations that Bcl-2 family members are known to dimerize. Thus the active principle in the SCF-containing fraction may itself be a Bcl-2 family member.

Given that reaper is itself only 65 amino acids, it was possible to chemically synthesize a peptide of the full reaper protein. This was done to simplify purification of

SCF, as the recombinant reaper protein generated from bacteria is quite impure. We sought to minimize the complexity of the SCF mixture, and so characterized the reaper peptide for its ability to induce apoptosis in the extract of Xenopus eggs (Fig. 5). As shown in Fig. 5, similar to the bacterially-expressed reaper protein, synthetic reaper peptide is able to induce caspase activation, as measured by cleavage of the colorimetric caspase substrate DEVD.pNA. Unfortunately, the peptide proved useless for SCF characerization, as it induced the release of cytochrome c from purified mitochondria in the absence of other factors (unlike bacterial reaper). This cytochrome c release is likely due to the chemical disruption of the outer mitochondrial membrane, as it takes place immediately upon addition of the peptide to the mitochondria.

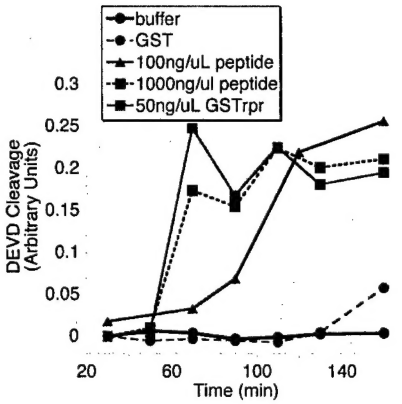

Fig. 5: Synthetic Reaper peptide is active in crude extract

Further characterization of the peptide was interrupted by observations made in a parallel effort in the lab, by another graduate student, who identified scythe as a member of the BAG family of chaperone inhibitors. Scythe, like BAG-1 and other members of the BAG family, inhibit chaperone function by binding and preventing ATP hydrolysis of Hsp70. Moreover, following incubation with reaper, Hsp70 was released from scythe into the soluble fraction (Thress et al., 2001). Inhibiting chaperone function globally in the extract prevented reaper-induced apoptosis (Thress et al., 2001). A series of studies was then undertaken to see if Hsp70 could be involved in SCF activity.

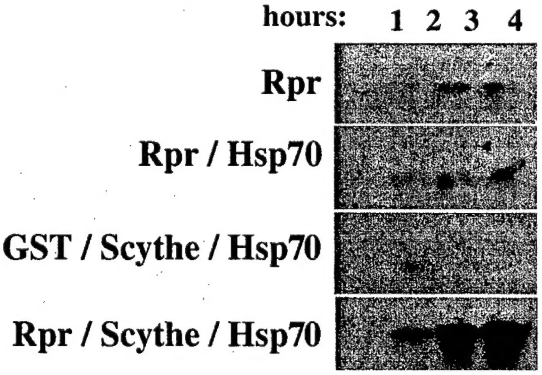

Fig. 6: Reconstitution of cytochrome c release

The first such experiment involved taking reaper, Hsp70, and scythe (with various combinations) and incubating them with purified mitochondria in an effort to reconstitute SCF activity. Interestingly, Hsp70 alone is insufficient to induce cytochrome c release (see Fig. 6). Yet when Hsp70, reaper, and scythe are co-incubated with mitochondria, a robust release of cytochrome c is generated, with similar kinetics to that seen in the crude extract (see Fig. 6). As shown in crude extract (Thress et al., 2000) inhibition of chaperone activity, accomplished by addition of the chaperone-inhibiting BAG domain, blocks cytochrome c release. We are left then with a model (see Fig. 7) in which reaper association with Scythe liberates the chaperone protein Hsp70. Hsp70 then must be required to fold a substrate at the mitochondrial surface which then triggers cytochrome c release. Scythe and reaper are presumably involved in targeting Hsp70 to its substrate, and dis-inhibiting its activity, respectively.

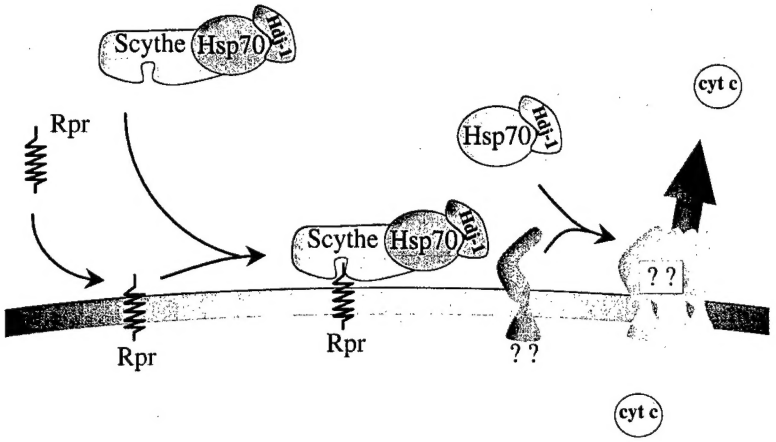

Fig.7: Model of Hsp70 action

Finally, given the importance of Bcl-2 family members to the release of cytochrome c in other systems, my identification of a putative Bcl-2 domain within scythe itself was of particular interest (see Fig. 8). Mutants of scythe were made in which this domain was altered to knock out Bcl-2-like function. Nevertheless, both mutant and wild-type scythe was fully functional in the reconstitution assay (see Fig. 9).

bcl-2 bh3	V	H	L	T	L	R	R	A	G	D	D	F
bcl-xl bh3	V	K	Q	A	L	R	E	A	G	D	E	F
bax-a bh3	L	S	E	C	L	K	R	I	G	D	E	L
bak bh3	V	G	R	Q	L	A	I	I	G	D	D	I
bad bh3	Y	G	R	E	L	R	R	M	S	D	E	F
bim bh3	I	A	Q	E	L	R	R	I	G	D	E	F
bid bh3	I	A	R	H	L	A	Q	V	G	D	S	M
Noxa BH3, b	E	C	A	Q	L	R	R	I	G	D	K	V
Noxa BH3, a	F	A	A	Q	L	R	K	I	G	D	K	V
p193 BH3	V	V	R	I	L	K	A	H	G	D	E	G
Scythe BH3	I	Q	R	H	V	R	R	V	G	D	A	P
Scythe BH3mut	I	Q	R	H	A	R	R	S	R	R	A	P

Fig.8: BH3 domain of Scythe

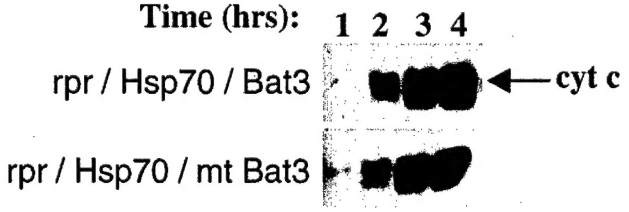

Fig.9: BH3 domain of Scythe

In summary, the vast majority of Task 1 has been completed. We have preliminarily identified SCF as an Hsp70 substrate which is most likely a Bcl-2 family member. Good candidates for this protein include Bax and Bak, two Bcl-2 family members which are known to become cytochrome c-releasing following a conformational change (potentially inducible by Hsp70). Efforts to confirm this are now ongoing. We have ruled out both use of the reaper peptide in indentification of SCF, and involvement of the scythe BH_3-like domain in SCF activity. Given our targeted approach to SCF identification, it is likely that neither microsequencing nor cloning of SCF will be required, although these are still possibilities. Should the targeted approach fail, we have made excellent strides in the biochemical purification of SCF, and can continue with this as necessary.

As part of the effort to characterize the reaper peptide (see above), experiments were done investigating the effect of reaper on IAP stability. These experiments formed

the basis for a manuscript published this year in Nature Cell Biology (Holley et al., 2002). Furthermore, as part of the effort to characterize SCF, mutants of reaper have been generated which are defective in their ability to effect apoptosis. These mutants will form the basis of another publication still in progress.

KEY RESEARCH ACCOMPLISHMENTS

- SCF binds to and elutes from DEAE resin
- SCF binds to Bcl-XL, and is therefore likely a Bcl-2 family member
- SCF activity requires Hsp70 chaperone activity
- SCF activity can be reconstituted with Hsp70, Scythe, Reaper, and purified mitochondria
- Bcl-2 domain of scythe is not involved in SCF activity
- Synthetic reaper peptide, while of no use in elucidating SCF activity, was useful in examining the effects of reaper on IAP half-life (Holley et al., 2002).

REPORTABLE OUTCOMES

Papers to date:

Holley, C.*, Olson, M.R.*, Colon-Ramos, D.A.*, Kornbluth, S. (2002) *NCB* **4**. 439-44. (* = equal contributors)

CONCLUSIONS

SCF is likely to be a Bcl-2 family member being activated on by Hsp70 which has been released from scythe in response to reaper. Further studies will be required (tasks 3 and 4 of Statement of Work) to determine whether SCF will remain active in the setting of a transformed cell line. Given the published efficacy of reaper in the killing of MCF-7 breast carcinoma cells (McCarthy and Dixit, 1999), it will be interesting to see how reaper overcomes the Bcl-2 protection present in this cell line.

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APPENDICES

Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition

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focus on *apoptosis*

Inhibitors of apoptosis (IAPs) inhibit caspases, thereby preventing proteolysis of apoptotic substrates. IAPs occlude the active sites of caspases to which they are bound[1–3] and can function as ubiquitin ligases. IAPs are also reported to ubiquitinate themselves and caspases[4,5]. Several proteins induce apoptosis, at least in part, by binding and inhibiting IAPs. Among these are the *Drosophila melanogaster* proteins Reaper (Rpr), Grim, and HID, and the mammalian proteins Smac/Diablo and Omi/HtrA2, all of which share a conserved amino-terminal IAP-binding motif[6–14]. We report here that Rpr not only inhibits IAP function, but also greatly decreases IAP abundance. This decrease in IAP levels results from a combination of increased IAP degradation and a previously unrecognized ability of Rpr to repress total protein translation. Rpr-stimulated IAP degradation required both IAP ubiquitin ligase activity and an unblocked Rpr N terminus. In contrast, Rpr lacking a free N terminus still inhibited protein translation. As the abundance of short-lived proteins are severely affected after translational inhibition, the coordinated dampening of protein synthesis and the ubiquitin-mediated destruction of IAPs can effectively reduce IAP levels to lower the threshold for apoptosis.

To evaluate the effects of Rpr on the function of IAPs, we cotransfected human 293T cells with untagged Rpr and human members of the IAP family: XIAP and cIAP1. In the presence of Rpr, IAP steady-state levels were much lower than in the presence of vector alone, suggesting that Rpr was preventing XIAP and cIAP1 protein accumulation (Fig. 1a). Similar results were obtained in fly embryos, where overexpression of Rpr resulted in barely detectable levels of DIAP1 (B. Hay, personal communication). Note that 'laddered' forms of XIAP, indicative of ubiquitination, were recognized by anti-ubiquitin antibody (Fig. 1b), consistent with previous reports of IAP auto-ubiquitination[4,5].

We therefore hypothesized that Rpr might stimulate IAP ubiquitination and degradation. To determine whether Rpr affects IAP half-life, we performed pulse-chase analyses on cells cotransfected with XIAP and either Rpr or vector alone. Cotransfection with Rpr significantly affected XIAP stability (Fig. 1c; see also Fig. 3b). Moreover, Rpr greatly increased the appearance of laddered XIAP species. This change in IAP stability was not a consequence of Rpr-induced apoptosis, as the pulse-chase experiments were performed in the presence of the broad-spectrum caspase inhibitor zVAD-fmk.

To address the effects of Rpr on IAP stability in an alternative system, we examined the half-lives of radiolabelled human IAPs added to whole-cell lysates prepared from *Xenopus laevis* eggs, which reconstitute both apoptotic signalling and ubiquitin-dependent proteolysis[15,16]. Radiolabelled, *in vitro* -translated cIAP1 and XIAP proteins were added to egg extracts supplemented with vehicle or with full-length, untagged Rpr, prepared by complete *de novo* peptide synthesis[17]. As in cultured cells, Rpr addition to egg extracts significantly destabilized both cIAP1 and XIAP (Fig. 1d). Similar results were obtained with a fly IAP, DIAP1 (Fig. 4c).

To extend these findings, we isolated a *Xenopus* XIAP homologue, XLX. Domain analysis of XLX revealed two complete and one partial N-terminal baculovirus inhibitory repeat (BIR) domain, and a carboxy-terminal RING domain (Fig. 2a). In common with XIAP, XLX lacks the caspase activation recruitment domain (CARD) found in cIAP1 and cIAP2 (ref. 18). Despite truncation of BIR domain 1 in our clone, we believe XLX to be full-length, as the cDNA isolated contains three in-frame stop codons within the 5′-untranslated region (UTR) preceding the start methionine.

Because IAPs can be caspase substrates (Fig. 2b), the disappearance of IAPs in our extracts might have been caused, at least in part, by caspase-mediated cleavage[19,20]. In fact, glutathione *S*-transferase (GST)–Rpr induces mitochondrial cytochrome *c* release, thereby activating caspases in the extract[21]. Similarly, addition of Rpr peptide to crude *Xenopus* egg extracts triggered caspase activation, although at the concentration used in our IAP experiments (100 ng μl^{-1}), caspase activation was relatively delayed (Fig. 2c). However, as reported for GST–Rpr[21], the Rpr peptide could not induce caspase activation in egg cytosol lacking mitochondria (Fig. 2d; note caspase activation by cytochrome *c* addition to the same extract). Nevertheless, in these cytosolic extracts, the Rpr peptide significantly accelerated the destruction of XLX (Fig. 2e,f). XLX cleavage fragments were absent in these extracts (Fig. 2e, arrowheads) and in crude extracts incubated with zVAD-fmk (data not shown). Therefore, although caspases can cleave XLX, they are not essential for Rpr-accelerated IAP destruction. In contrast to the Rpr peptide, GST–Rpr (whose IAP-binding N terminus is shielded by its GST tag), failed to accelerate XLX destruction (Fig. 2e,f). These data suggest that Rpr-stimulated degradation of IAPs can occur independently of caspase activation, and that this effect requires the N terminus of Rpr to be unblocked. Consistent with the hypothesis that Rpr requires a free N terminus to promote IAP degradation, GST–Rpr and XIAP did not co-precipitate (Fig. 3a, right). In addition, an untagged Rpr lacking amino acids 1–15 (Rpr^{16-65}) could not promote IAP degradation, further demonstrating that the extreme N terminus of Rpr is required to shorten IAP half-life (Fig. 3b).

To determine whether IAP ubiquitin ligase activity was required for Rpr-induced IAP degradation, we cotransfected cells with Rpr and a catalytically inactive XIAP point mutant of $XIAP^{H467A}$ (ref. 4;

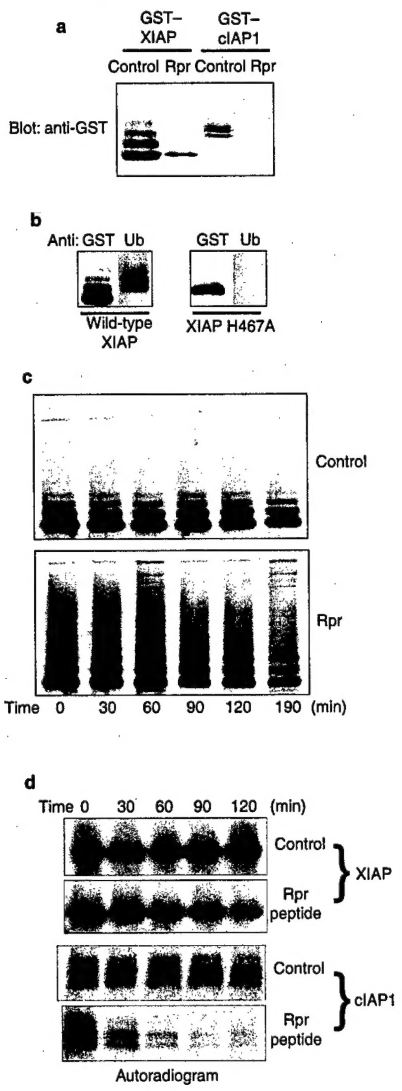

Figure 1 **Rpr stimulates IAP auto-ubiquitination and destruction. a**, HEK 293T cells were transfected with GST–XIAP or GST–cIAP1 and either Rpr or vector control, in the presence of zVAD-fmk. Following precipitation with glutathione–Sepharose, GST fusion protein levels were analysed by immunoblotting with an anti-GST polyclonal antibody. **b**, HEK 293T cells were transfected with either wild-type ubiquitin ligase or an XIAP^{H467A} mutant and precipitated with glutathione–Sepharose. Precipitates were immunoblotted with an antibody against GST or ubiquitin. **c**, HEK 293T cells were transfected with GST–XIAP and either Rpr or vector control. Cells were then radiolabelled in a pulse-chase experiment. The resulting radiolabelled GST fusion proteins were analysed by autoradiography. **d**, Radiolabelled IAPs were incubated in crude *Xenopus* egg extracts, in the presence of either Rpr peptide or vehicle control. The resulting radiolabelled protein levels were analysed by autoradiography. Equal loading was verified by Coomassie blue staining of gels (data not shown).

see also Fig. 1b). Although Rpr bound to XIAP^{H467A} (Fig. 4a), it failed to accelerate destruction of the mutant in a pulse-chase experiment (Fig. 4b). Rpr had similar effects on its *Drosophila* target, DIAP1. Again, destabilization was dependent on an intact RING domain, as the DIAP1 ubiquitin ligase mutant DIAP1^{C412Y} was not significantly destabilized (Fig. 4c). Collectively, these data demonstrate that Rpr-stimulated IAP degradation requires that the IAP be functional as a ubiquitin ligase.

Although untagged, full-length Rpr substantially destabilized all of the wild-type IAP proteins tested, we were surprised to find that Rpr also moderately decreased steady-state levels of an unrelated protein after cotransfection of human cells (Fig. 5a, GST). Additionally, overexpression of Rpr in flies lowers the levels of a DIAP1 ubiquitin ligase mutant, implying that Rpr has effects *in vivo* that are independent of its effects on IAP half-life (B. Hay, personal communication). This prompted us to examine whether IAP abundance might also be affected at the level of protein production. Indeed, when we programmed reticulocyte lysates with XIAP or XLX, IAP levels were profoundly decreased by GST–Rpr and essentially eliminated by the Rpr peptide (Fig. 5b). GST, or other unrelated proteins, had no effect (Fig. 5b and data not shown). These effects on IAP levels were not caused by IAP degradation, as GST–Rpr failed to alter IAP levels when added to reticulocyte lysates, after translation had been blocked with cycloheximide (Fig. 5b).

Because the IAP constructs used in the reticulocyte lysates lacked native 5′-or 3′-UTR sequences, we considered it unlikely that the degradation-independent effects of Rpr were IAP-specific. Accordingly, when reticulocyte lysates were programmed with unrelated messages, the GST–Rpr protein also effectively dampened their expression (Fig. 5c). Again, this effect was not caused by protein degradation, as GST–Rpr addition did not affect levels of previously transcribed and translated proteins in reticulocyte lysates (Fig. 5c).

To assess the effects of Rpr on total protein synthesis, we added GST–Rpr to *Xenopus* egg extracts, which were translationally competent and transcriptionally inactive. These extracts were supplemented with ^{35}S-Met/Cys and high levels of zVAD-fmk to prevent caspase-mediated cleavage of translation factors. Addition of GST–Rpr or Rpr peptide to *Xenopus* egg extracts globally suppressed protein synthesis (Fig. 5d,e). Importantly, unrelated GST fusion proteins prepared in the same manner as GST–Rpr had no such effect (Fig. 5d,e). Rpr did not reduce protein levels by accelerating general protein degradation, as co-addition of GST–Rpr or Rpr peptide and cycloheximide to extracts after 45 min of translation did not result in destruction of nascent proteins (Fig. 5d,e). These data strongly suggest that the ability of Rpr to post-translationally destabilize proteins is specific to the IAPs. Thus, Rpr can decrease generalized translation in a manner distinct from its ability to accelerate the ubiquitin-mediated destruction of extant IAPs. Unlike the effect on IAP protein stability, the Rpr effect on translation did not require a free N terminus, as GST–Rpr was effective in translational inhibition. GST–Rpr$^{16-65}$, which lacks the first 15 amino acids of Rpr, also inhibited translation, confirming that the extreme N terminus of Rpr is dispensable for translational inhibition (Fig. 5e).

Although GST–Rpr was able to decrease IAP levels (Fig. 5b), the Rpr peptide was more effective in this regard. We hypothesized that the peptide might more effectively lower wild-type XIAP protein levels by simultaneously shortening XIAP half-life and inhibiting protein translation. We therefore returned to the reticulocyte lysate system to examine levels of the XIAP^{H467A}, as this mutant is not subject to Rpr-mediated degradation. When the XIAP mutant was examined in this system, we found that GST–Rpr indeed suppressed translation of this protein, as it had with other proteins tested. However, whereas the abundance of wild-type XIAP had been more dramatically reduced by the Rpr peptide than by GST–Rpr, the abundance of the XIAP ubiquitin ligase mutant was suppressed equally by both (compare Fig. 5b and f).

Despite the robust translational inhibition by Rpr *in vitro*, we wanted to determine whether we could detect such effects of Rpr in

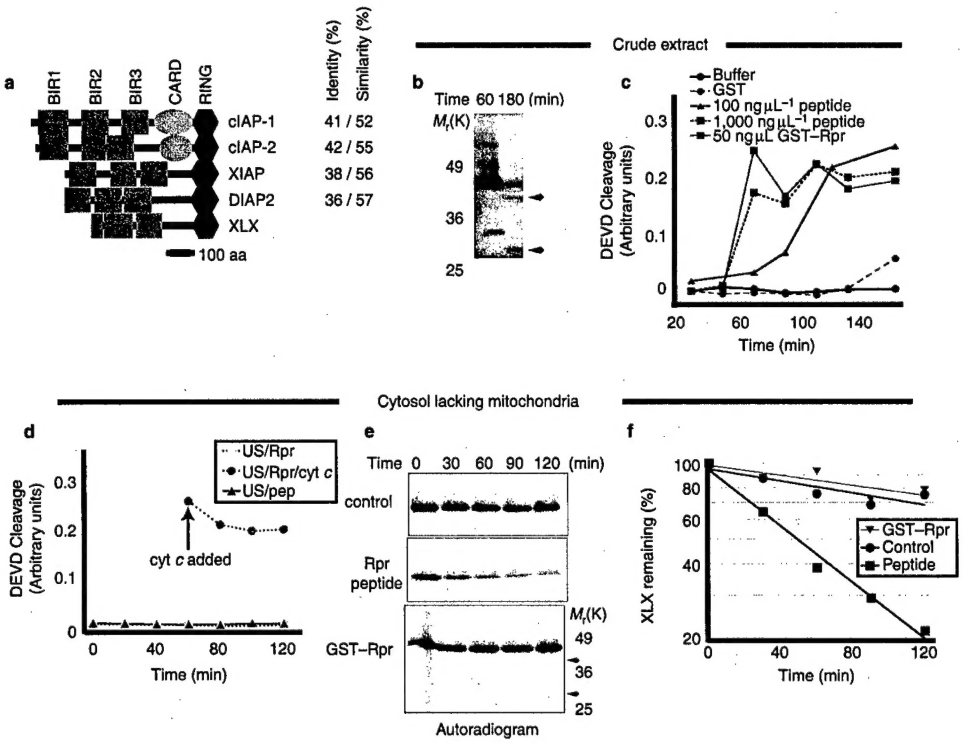

Figure 2 **XLX, a *X. laevis* XIAP homologue, is destabilized by Rpr peptide, but not by GST–Rpr. a**, A domain map drawn to the scale of XLX with three human IAPs: cIAP1, cIAP2, and XIAP, and *D. melanogaster* DIAP2 is shown. Grey boxes represent BIR domains, ovals represent caspase recruitment domains (CARD), and hexagons represent C-terminal RING domains. The percentage identity and similarity between residues of each protein to XLX are indicated. BLAST alignment of XLX to proteins in the non-redundant public database yielded chicken and human XIAP as the most similar clones. XLX has a similar domain structure to XIAP and DIAP2. **b**, GST–Rpr and radiolabelled XLX were added to crude *Xenopus* egg extract. Samples were resolved by SDS–PAGE and autoradiography at the indicated times. Arrowheads denote the ~40K and 30K XLX cleavage products. Molecular weight markers are shown for reference with **e** below. **c**, GST–Rpr, GST, Rpr peptide or peptide vehicle were added to crude *Xenopus* egg extract. Caspase activity was monitored by cleavage of the colorimetric peptide substrate DEVD–pNA. **d**, GST–Rpr (Rpr) or Rpr peptide (Pep) were added to *Xenopus* egg extract depleted of mitochondria by centrifugation (US). At 60 min, human cytochrome *c* was added to an aliquot of the extract containing GST–Rpr (arrow). Caspase activity was monitored as in **c** above. **e**, Rpr peptide, peptide vehicle (control) or GST–Rpr were added together with radiolabelled XLX into *Xenopus* egg extract depleted of mitochondria. Samples were taken at the indicated times and XLX protein levels were analysed by SDS–PAGE and autoradiography. Molecular weight markers are shown for comparison with **b** and arrows indicate the approximate position of expected XLX cleavage fragments (which are absent). Equal loading was verified by Coomassie blue staining of gels (data not shown). **f**, The autoradiogram in **e** was quantified using a phosphorimager.

intact cells. Accordingly, we injected whole *Xenopus* oocytes with zVAD-fmk and either *rpr* sense or anti-sense mRNA. After 12 h incubation to allow translation of the Rpr protein, we re-injected oocytes with ^{35}S-methionine, incubated them for a further 4 h, lysed the oocytes and assessed the level of total protein synthesis by measurement of TCA-precipitable radioactivity. The oocytes injected with zVAD-fmk and *rpr* sense mRNA incorporated approximately sevenfold less counts than the anti-sense controls (~1.1×10^5 cpm versus ~7.9×10^5 cpm). These data demonstrate that even when synthesized *de novo* within an intact cell, Rpr can inhibit protein translation. Consistent with these results, cotransfection of human cells with Rpr and GST reduced GST synthesis by ~30% in a pulse labelling experiment, despite the very low levels of Rpr produced in these cells (data not shown). Although these results were more modest than those obtained in reticulocyte lysates or oocytes, we have not been able to achieve comparable levels of Rpr in the intact tissue culture cells. However, even a moderate reduction in protein synthesis, coupled with a decrease in IAP stability, would synergize to produce an effective elimination of the IAPs.

In aggregate, our data suggest that Rpr eliminates IAPs by simultaneously stimulating their ubiquitin-mediated degradation and down-regulating total protein translation. This reduction in IAP levels by Rpr lowers the threshold for caspase function, thereby facilitating apoptotic progression.

Note added in proof: Several other papers in this issue also demonstrate that Reaper functions to stimulate IAP degradation[23–25]. *Additionally, another paper in this issue supports our findings that Reaper suppresses general protein translation*[26]. □

Methods

Cell culture, transfections, immunoblotting, and pulse-chase analysis

All cell culture reagents were obtained from Gibco (Rockville, MD) unless otherwise specified. HEK 293T cells were obtained from the American Type Culture Collection (ATCC) through the Duke Cell Culture Facility, and were maintained in MEM, which was supplemented with 10% foetal bovine serum, 1 mM sodium pyruvate and 0.1 mM MEM non-essential amino acids solution. The *Drosophila rpr* gene was cloned into pEBB using standard methods. For the immunoblots shown, 1×10^6 cells were plated in 100-mm dishes and transfected 24 h later using the Fugene 6 reagent (Roche Molecular, Indianapolis, IN) and 10 μg of total DNA, according the manufacturer's instructions. 24–48 h after

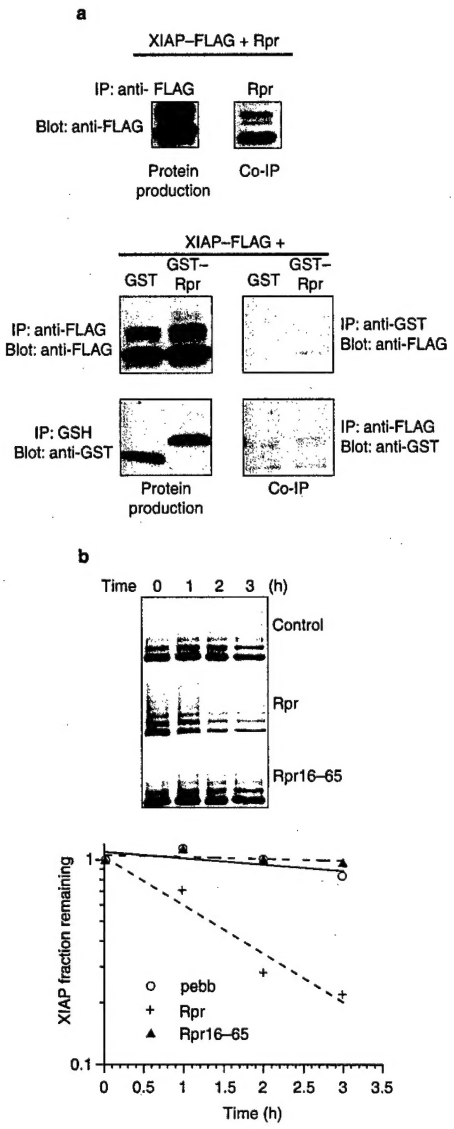

Figure 3. **An unblocked Rpr N terminus is required for IAP binding and stabilization. a**, HEK 293T cells were transfected with Rpr, GST alone or GST–Rpr and FLAG-tagged XIAP protein. Proteins were precipitated with anti-FLAG or anti-Rpr antibodies and blotted with anti-FLAG. Proteins were precipitated with glutathione–Sepharose or anti-FLAG, and detected by immunoblotting with anti-GST or anti-FLAG. Although GST–Rpr and XIAP failed to coprecipitate, the untagged Rpr control clearly coprecipitated with XIAP protein. **b**, A pulse-chase experiment was performed as in Fig. 1c, using either wild-type Rpr (Rpr) or untagged Rpr lacking its first 15 amino acids (Rpr^{16-65}). The results were then quantified.

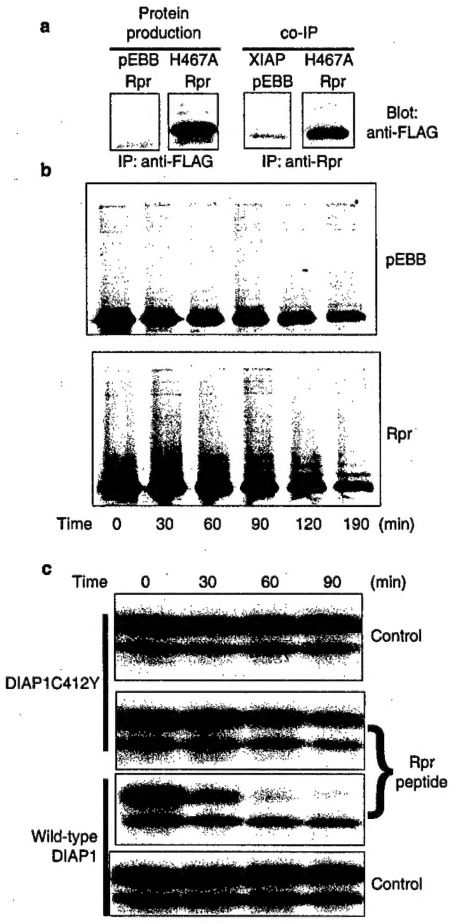

Figure 4. **Rpr does not destabilize the $XIAP^{H467A}$ ubiquitin ligase mutant. a**, HEK 293T cells were transfected with FLAG-tagged wild-type XIAP or FLAG-tagged catalytically inactive XIAP ($XIAP^{H467A}$) in conjunction with a control vector (pEBB) or untagged Rpr (Rpr). Samples were precipitated with an anti-FLAG antibody (left) or an anti-Rpr antibody (right), demonstrating a nearly quantitative association of the mutant XIAP with Rpr. **b**, HEK 293T cells were transfected with GST–$XIAP^{H467A}$ and either Rpr or vector control. Cells were then radiolabelled in a pulse-chase experiment, and the resulting radiolabelled GST–$XIAP^{H467A}$ proteins were analysed by autoradiography. **c**, Radiolabelled DIAP1 protein, either wild-type or the catalytically inactive $DIAP^{C412Y}$ mutant, was incubated in *Xenopus* egg extract lacking mitochondria, in the presence of Rpr peptide or vehicle control. The resulting radiolabelled protein levels were analysed by autoradiography.

transfection, cells were washed once in PBS, collected in lysis buffer (10 mM HEPES at pH 7.4, 50 mM potassium chloride, 2.5 mM magnesium chloride and 50 mM sucrose, plus 1× Complete protease inhibitor (Roche Molecular)) and briefly sonicated. Lysates were incubated for 10 min on ice and cleared by centrifugation at 10,000*g* for 10 min. Cleared lysates were then incubated with glutathione–Sepharose (Pharmacia) or the M2 anti-FLAG antibody (Sigma, St Louis, MO) and Protein G–agarose (Oncogene Research Products, Boston, MA) or K1 anti-Rpr antibody and Protein A–Sepharose (Sigma) at 4 °C for 1 h. The bead-bound material was washed three times in lysis buffer and released in 2×SDS sample buffer. This material was then separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes by standard methods. Membranes were blocked in PBS containing 0.1% Tween-20 and 5% dry milk. For immunoblotting to detect GST fusions, rabbit antiserum to GST was used at 1:3,000 in PBS containing 0.1% Tween-20 and 2% BSA, before incubation with Protein A–horseradish peroxidase (HRP; Amersham, Sunnyvale, CA) at 1:10,000. Immunoblots to detect FLAG-tagged proteins were handled similarly using the M2 anti-FLAG antibody (1 μg $μl^{-1}$) and goat anti-mouse-HRP (Jackson ImmunoResearch, West Grove, PA), whereas ubiquitin was detected using mouse anti-ubiquitin (1:100; Zymed, San Francisco, CA) and Protein A–HRP without pre-blocking the membrane. Blots were developed using Renaissance ECL reagents (NEN, Boston, MA) and exposed to Biomax ML film (Kodak, Rochester, NY). For pulse-chase analysis, 200,000 cells were plated per well in 6-well plates and transfected as above, except that a total of 1.5 μg DNA was used. 16–20 h after transfection, cells were washed once in prewarmed pulse medium (DMEM minus L-Met and L-Cys supplemented with 10% dialysed foetal bovine serum and 1 mM sodium pyruvate) and then incubated for 15 min in pulse medium to deplete Met and Cys levels. Cells

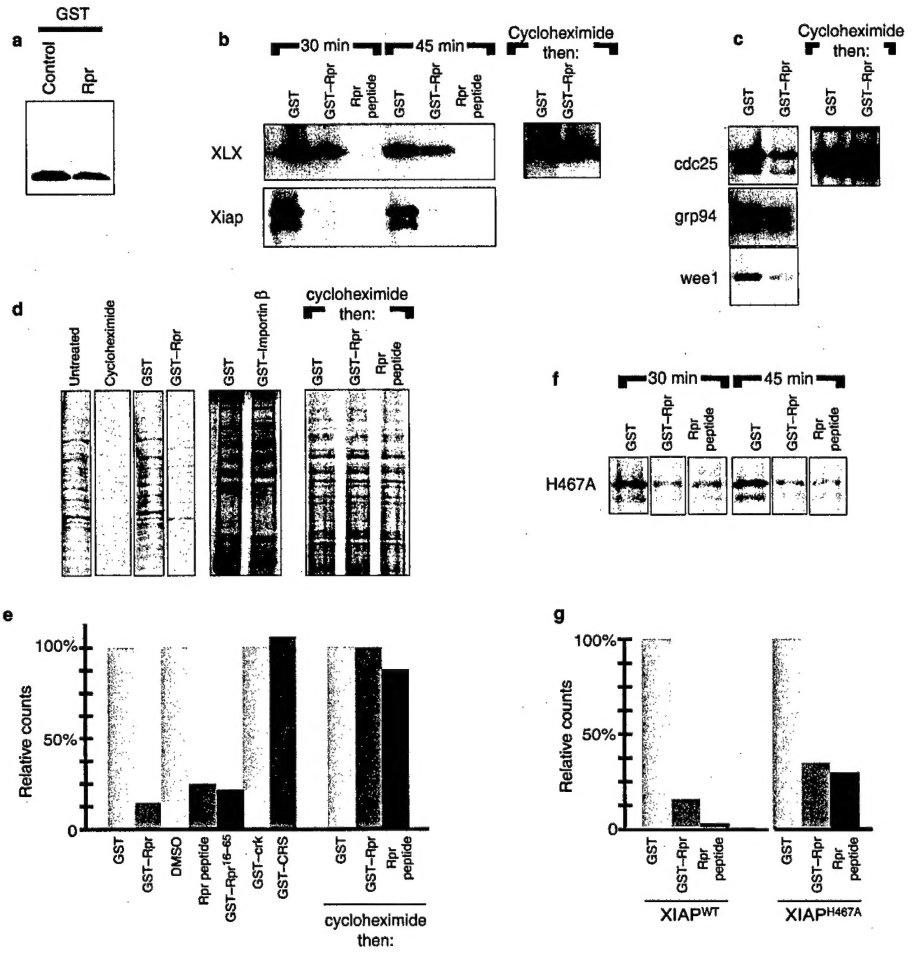

Figure 5 **Repression of translation by GST–Rpr and Rpr peptide. a**, HEK 293T cells were transfected with GST and either Rpr or vector control. Resulting GST protein levels were analysed by immunoblotting. **b**, XLX and human XIAP were added to rabbit reticulocyte lysates, which were supplemented with GST, GST–Rpr or Rpr peptide. Aliquots were collected at 30 and 45 min, and resolved by SDS–PAGE (left). Rabbit reticulocyte lysates were programmed with XLX and allowed to transcribe and translate for 45 min. Translation was stopped with the addition of cycloheximide and the lysates were incubated for an additional hour with GST or GST–Rpr before resolution by SDS–PAGE (right). **c**, Cdc25, Grp94 and Wee1 cDNAs were added to reticulocyte lysates and supplemented with GST or GST–Rpr, as above (left). The rabbit reticulocyte lysates were also allowed to transcribe and translate for 45 min, after which translation was stopped with cycloheximide (right). Samples were then incubated for an additional hour with GST or GST–Rpr, as above. **d**, Translationally competent, but transcriptionally inactive, *Xenopus* egg extracts were supplemented with ^{35}S-Met/Cys and zVAD-fmk plus egg lysis buffer (Untreated), cycloheximide, GST, GST–Rpr, or GST–importin-β as a non-specific control (left). Translation was allowed to proceed for 45 min and products were resolved by SDS–PAGE. Egg extracts supplemented with ^{35}S-Met/Cys and zVAD-fmk were allowed to translate for 45 min, translation was stopped with cycloheximide and extracts were incubated for an additional hour with GST, GST–Rpr or Rpr peptide (right). **e**, Samples prepared as in **d** were subjected to TCA precipitation and quantified by scintillation counting. Additionally, control proteins (GST–cyclin B1, GST–CRS, GST–Crk), peptide vehicle (DMSO), GST–Rpr$^{16–65}$ or Rpr peptide were assayed in the same manner. The resulting incorporated counts were TCA precipitated and scintillation counting was performed as above. **f**, XIAP^{H467A}, a mutant unable to function as a ubiquitin ligase, was added to rabbit reticulocyte lysate, which was supplemented with GST, GST–Rpr or Rpr peptide, and aliquots were collected at 30 and 45 min before resolution by SDS–PAGE. **g**, Phosphorimager quantification of wild-type XIAP and XIAP^{H467A} protein levels from **b** and **f**.

were then radiolabelled for 30 min with pulse medium containing 200 μCi ml^{-1} of ^{35}S-Trans label (ICN, Costa Mesa, CA). After labelling, cells were washed once with their normal culture medium and incubated in the complete medium for the chase times indicated. Radiolabelled proteins were harvested by rinsing the cells once in PBS and then lysing in 0.1% NP40, 150 mM sodium chloride, 50 mM HEPES at pH 7.4 and 1 mM EDTA, plus 1× protease inhibitors as above. Cell lysates were cleared by incubation on ice and centrifugation as above. GST–fusion proteins were captured on GSH–Sepharose and separated by SDS–PAGE as above. Gels were soaked in 1 M salicylate (Sigma) for 30 min before drying and overnight exposure to Biomax MR film (Kodak).

Cloning of XLX

A probe derived from the RING domain of human cIAP1 was generated using the Random Primed Labelling kit (Roche Molecular) and used to screen ~500,000 clones of a λ-zap *Xenopus* gastrula library at low stringency. Several clones >1 kB were isolated, excised and partially sequenced. A secondary screen was performed for one of the clones isolated using oligonucleotides designed to anneal to the linker region between the BIR and RING domains. The probe was generated by PCR with radiolabelled nucleotides (oligonucleotides: 5′-GATCTTTAGAAGCCCAGAGTCCTCTCCT-3′ and 5′-GATCCTTGCTCTGAATTAGACTTGCCAC-3′). This screen failed to isolate any larger clones. The

~1.6 kB cDNA was fully sequenced and deposited in GenBank. A BLAST alignment was performed using both the complete cDNA and the longest uninterrupted open reading frame. Domain analysis was performed using InterPro (http://www.ebi.ac.uk/interpro/).

Extract preparation

Preparation of crude interphase egg extracts (CS) was performed as previously described[21]. To fractionate the crude egg extract into cytosolic (US) and membranous components, the crude extract was centrifuged further at 200,000*g* for 1 h in a Beckman TLS-55 rotor using a TL-100 centrifuge. The cytosolic fraction (ultra-S or US) was removed and recentrifuged for an additional 25 min at 200,000*g*. These reconstituted extracts were supplemented with an energy regenerating system consisting of 2 mM ATP, 5 mg ml^{-1} creatine kinase, and 20 mM phosphocreatine (final concentrations).

Production of GST, GST–Rpr and Rpr peptide

GST and GST–Rpr were prepared as previously described[21]. Rpr was also generated as a full-length, untagged synthetic peptide by B. Kaplan (City of Hope, Beckman Research Institute). The peptide was received as a lyophilized powder, which was stored solid at 4 °C. Before use, the peptide was resuspended in dimethylsulphoxide (DMSO) at 10 mg ml^{-1}, and then diluted to 1 mg ml^{-1} in egg lysis buffer (10 mM HEPES at pH 7.4, 50 mM potassium chloride, 2.5 mM magnesium chloride, 50 mM sucrose and 1 mM dithiothreitol (DTT)).

DEVD assay

Recombinant GST, GST–Rpr, Rpr peptide or peptide vehicle (10% v/v DMSO in egg lysis buffer) was added at a 1:10 dilution to CS or US extract containing energy regenerating mix (see above). At the indicated times, 3-µl aliquots were withdrawn and incubated with 90 µl of assay buffer (50 mM HEPES at pH 7.5, 100 mM sodium chloride, 0.1% CHAPS, 10 mM DTT, 1mM EDTA and 10% glycerol) containing 200 µM Ac–DEVD pNA colorimetric substrate (BioMol, Plymouth Meeting, PA). Caspase-3 activity was monitored by the measurement of absorbance at 405 nm using a LabSystems MultiSkan MS microtiter 96-well plate reader (Helsinki, Finland).

In vitro translation

XIAP ORFs were subcloned using standard techniques into pBS II-SK and pSP64T, a TNT expression vector with flanking 5′ and 3′ β-globin UTR and a polyadenosine tail. To produce radioactive protein for half-life assays, Cdc 25, Grp94, Wee1, XLX, wild-type XIAP/DIAP, and $XIAP^{H467A}$/$DIAP^{C412Y}$ templates were added at 20 ng $µl^{-1}$ to rabbit reticulocyte lysate (Stratagene, La Jolla, CA) containing 1 µCi $µl^{-1}$ of Trans label, 1× (minus-Cys, minus-Met) amino acid mix and other components, in accordance with the manufacturer's protocol. For *Xenopus* stability assays, the reaction was stopped after 90 min and proteins were snap frozen in liquid nitrogen for later use. For translation inhibition assays, reticulocyte lysate reactions were supplemented with 100 ng $µl^{-1}$ of recombinant GST or GST–Rpr proteins, or Rpr peptide. Aliquots were withdrawn at the indicated times, resolved by SDS–PAGE, quantified with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA), and exposed to film. Protein degradation was assayed by allowing translation to proceed for 45 min, at which point cycloheximide was added to a final concentration of 500 ng $µl^{-1}$. Subsequently, GST–Rpr, Rpr peptide or GST were added to a final concentration of 100 ng $µl^{-1}$ and the mixture was incubated for 60 min at room temperature. Translated proteins were resolved by SDS–PAGE and quantified with the phosphorimager as described above.

Xenopus extract stability assay

In vitro-translated proteins were added on ice at 1:10 dilution into 100 µl of either CS or US lysate (with energy mix) that had been supplemented with 100 ng $µl^{-1}$ GST, GST–Rpr, or Rpr peptide. Where indicated, zVAD-fmk (BioMol) or DMSO vehicle was also added at a final concentration of 100 µM (data not shown). Samples were shifted to room temperature or 4 °C and 20-µl aliquots were withdrawn at the indicated times, mixed with 40 µl SDS loading buffer and flash frozen in liquid nitrogen. Samples were thawed by boiling for 5 min and then assayed by SDS–PAGE before quantification on a phosphorimager and exposure to film.

Xenopus extract translation assay

In vitro translation assays using *Xenopus* extract were conducted by adding 1 µCi $µl^{-1}$ of Trans label, 100 µM zVAD-fmk and 100 ng $µl^{-1}$ of recombinant GST, GST–crk, GST–CRS (Cyclin B cytoplasmic retention sequence), GST–importin-β, GST–Rpr, GST–Rpr^{16-65} proteins or Rpr peptide to crude egg extract. The extent of protein translation was assayed by SDS–PAGE analysis and quantified by autoradiogram and phosphorimager, or by TCA precipitation (80 µg of extract in 20% TCA). Rpr-induced degradation was assayed as in reticulocyte lysates (above), save that in addition, total translated protein was also quantified by TCA precipitation as described.

Oocyte micro-injection and translation assay

Stage VI oocytes of *X. laevis* were prepared for micro-injection as described[22]. 25 nl of 0.4 µg $µl^{-1}$ sense or antisense *rpr* RNA produced using the mCAP RNA capping kit (Stratagene) were injected into oocytes along with 100 µM zVAD-fmk. *Rpr* expression was allowed to proceed overnight, before an injection of Trans Label (25 nl of 10 µCi $µl^{-1}$). 25 oocytes injected with *rpr* sense or antisense RNA were collected 4 and 5 h after Trans label injection. The oocytes were lysed in buffer (5 mM HEPES at pH 7.8, 88 mM sodium chloride, 1 mM potassium chloride, 1 mM magnesium sulphate, 2.5 mM $NaHCO_3$, 0.7 mM calcium chloride and 50 ng $µl^{-1}$ apropotein/leupetin/ cytochalasin B) by centrifugation at 16,000*g* for 15 min. Total protein translation was assayed by TCA precipitation (80 µg of oocyte extract in 20% TCA) as described.

Accession numbers

X. laevis XIAP (XLX) was submitted to GenBank and given the accession number AF468029.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.